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Human Neutrophil Fcγ Receptors Initiate and Play Specialized Nonredundant Roles in Antibody-Mediated Inflammatory Diseases

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SUMMARY

Inflammation mediated by antibody-antigen complexes contributes to autoimmune diseases. Mice deficient in the common Fcγ-chain are protected from IgG-mediated glomerulonephritis and the reverse passive Arthus (RPA) reaction and FcR-bearing macrophages, and mast cells have been assigned primary roles in these processes. Here we demonstrate that neutrophil-selective transgenic expression of the two uniquely human neutrophil Fc gamma receptors (FcγRs), FcγRIIA and FcγRIIIB, in Fcγ-chain-deficient mice restored susceptibility to progressive glomerulonephritis and the cutaneous RPA reaction. FcγRIIIB and FcγRIIA mediated neutrophil accumulation, whereas FcγRIIA alone promoted organ injury. In a model of soluble immune complexes deposited within the vasculature, FcγRIIIB was responsible for neutrophil slow rolling and adhesion whereas in the cremaster RPA, induced by both vascular and tissue soluble immune complexes, FcγRIIA predominated. Thus, human FcγRs on neutrophils serve as molecular links between antibody and immunological disease, with FcγRIIA promoting tissue injury and FcγRIIIB and FcγRIIA displaying specialized context-dependent functions in neutrophil recruitment.

INTRODUCTION

Deposition of antigen-antibody complexes in tissues is a hallmark of human diseases, from autoimmune disorders and early transplant rejection to rheumatic fever. IgG-mediated diseases are produced either by the binding of pathogenic antibody to self or foreign antigens on host cells or by the deposition of circulating antigen-antibody complexes in tissue. Cell-surface receptors that bind IgG-immune complexes (ICs), known collectively as Fcγ receptors (FcγRs), play essential roles in diseases initiated by antibodies (Ravetch and Bolland, 2001; Schmidt and Gessner, 2005). In particular, mice deficient in the common γ-chain (*Fcer1g*$^{-/-}$) required for expression of the murine activating FcγRs are protected in acute and progressive glomerulonephritis, autoimmune skin diseases, arthritis, systemic lupus erythematosus nephritis, and the reverse passive Arthus (RPA) reaction (Ji et al., 2002; Ravetch and Bolland, 2001; Trcka et al., 2002). In addition to FcγRs, C5aR binding to complement C5a activated by ICs may modulate disease pathogenesis by regulating the balance of FcγR expression and inducing chemokine release (Schmidt and Gessner, 2005). Despite the importance of FcγRs and complement proteins in IC-mediated inflammation, mechanisms downstream of their activation and the relevant Fc-bearing cell type involved still remain largely unresolved. The present view is that tissue-resident mast cells and macrophages sense ICs through FcγRs and complement receptors and, subsequently, release inflammatory mediators that recruit effector cells through the well-described multistep process of endothelial activation, selectin-dependent rolling, and integrin-mediated adhesion (Jancar and Sanchez Crespo, 2005; Ley et al., 2007; Schmidt and Gessner, 2005).

Neutrophils are key effector cells in innate immune responses, yet FcγRs specifically on neutrophils have not been implicated as initial mediators of cellular activation in IgG-disease models in mice. Given the structural differences between murine and human low-affinity FcγRs, it is not clear how well studies mediated by murine receptors accurately reflect human inflammation. Murine neutrophils express FcγRIII and FcγRIV (Nimmerjahn and Ravetch, 2006), which are transmembrane receptors relying on a common immunoreceptor tyrosine-based activation motif (ITAM)-containing γ-chain for expression and signaling. In contrast, human neutrophils express a unique glycosyl-phosphatidyl-inositol (GPI)-anchored FcγRIIIB and a single-polypeptide ITAM-containing FcγRIIA for which there are no genetic equivalents in mice or other mammals (Hogarth, 2002). Thus the low-affinity human receptors are single-polypeptide molecules, with FcγRIIA containing its own signaling domain, whereas the murine counterparts function as multiprotein complexes, with ligand-binding and signaling functions present on separate polypeptides.

The biological role of the two unique human neutrophil FcγRs, FcγRIIA and FcγRIIIB, remains largely unclear. Genetic evidence indicates that polymorphisms in FcγRIIA and IIIB correlate with autoimmune disease in patients (van Sorge et al., 2003), and a low gene copy number of FcγRIIIB predisposes systemic lupus erythematosus patients to glomerulonephritis (Aitman et al., 2006). In vitro, engagement of FcγRIIA promotes phagocytosis,

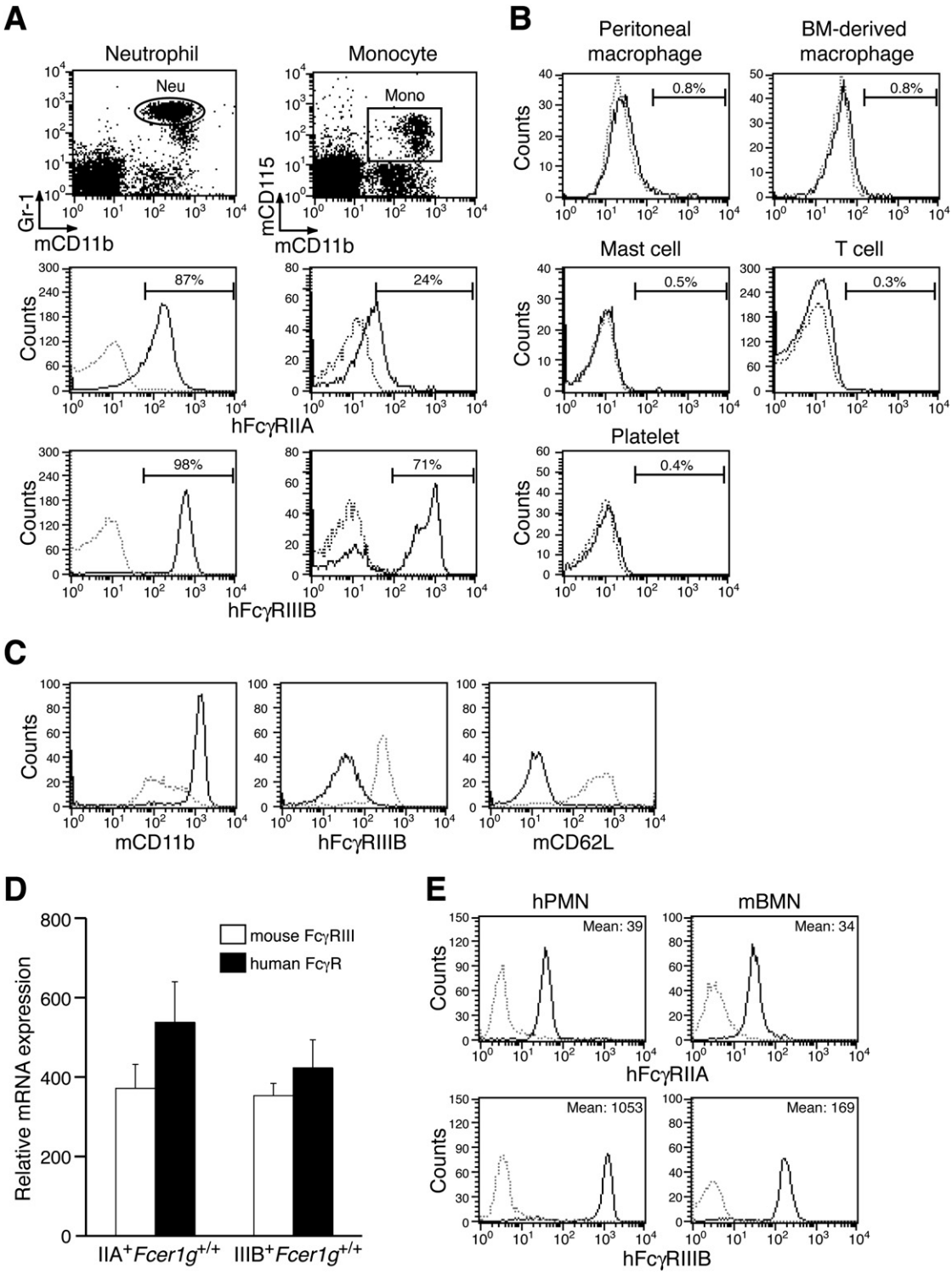

Figure 1. Neutrophil-Specific Expression of Human Fcγ Receptors in Transgenic Mice

(A and B) hFcγR expression was evaluated by flow cytometry on *Fcer1g*$^{-/-}$ (dotted line) and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ (solid line) mice. hFcγRIIA and IIIB expression was analyzed on (A) peripheral-blood neutrophils (Gr-1^{hi}mCD11b^{+}) and monocytes (mCD115^{+}mCD11b^{+}), and FcγRIIA expression was assessed on (B) F4/80^{+} resident peritoneal and bone marrow (BM)-derived macrophages, peritoneal mast cells, CD3^{+} T cells, and platelets. The percentages of FcγR-positive cells are indicated. (C) Cell-surface expression of mouse CD11b, hFcγRIIIB, and mouse CD62L (L-selectin) in bone-marrow neutrophils (BMN) from IIIB^{+} transgenic mice stimulated without (dotted line) or with 100 ng/ml of PMA for 10 min (solid line). hFcγRIIIB shedding (middle panel) is associated with the CD11b upregulation and L-selectin shedding, hallmarks of neutrophil activation.

degranulation, and reactive oxygen species (ROS) generation. FcγRIIIB's function remains elusive despite its 4- to 5-fold-higher surface expression compared to FcγRIIA in human neutrophils (Selvaraj et al., 1988; Unkeless et al., 1995). FcγRIIIB cross-linking induces calcium mobilization and triggers degranulation and leukotriene release (Crockett-Torabi et al., 1992; Unkeless et al., 1995). In vitro, human FcγRIIIB preferentially tethers neutrophils to immobilized ICs under physiological flow conditions (Coxon et al., 2001; Florey et al., 2007; Luscinskas and Mayadas, 2007), and FcγRIIA, in cooperation with chemokine receptors, was recently shown to enhance leukocyte adhesion to IgG bound to activated endothelial cells (Florey et al., 2007).

To examine the relative contribution of the human neutrophil-activating receptors FcγRIIA and FcγRIIIB in effector responses to IgG in vivo, we generated transgenic mice that express one or both of these receptors selectively in neutrophils by using a myeloid-restricted human migration inhibitory factor-related protein 8 (hMRP8, *S100A8*) promoter (Lagasse and Weissman, 1994; Lagasse and Weissman, 1997). These mice were crossed to *Fcer1g*$^{-/-}$ mice to eliminate endogenous murine activating receptors. We show that expression of both FcγRIIA and IIIB in neutrophils was sufficient to restore disease in *Fcer1g*$^{-/-}$ mice subjected to a model of progressive glomerulonephritis or RPA. These are prototypic models of type II and type III autoimmunity induced by in situ or soluble ICs, respectively. FcγRIIIB and FcγRIIA promoted neutrophil accumulation in both models, whereas FcγRIIA alone was required for tissue injury. The observed neutrophil recruitment in the absence of FcγR expression in macrophages and mast cells suggested a direct role for neutrophil FcγRs in neutrophil recruitment. We provide evidence that FcγRIIA and FcγRIIIB tether to in situ-formed glomerular ICs and play distinct context-dependent roles in soluble-IC-induced slow leukocyte rolling, adhesion, and transmigration. Together, our work suggests a paradigm in human IgG-mediated diseases wherein neutrophils are recruited and promote tissue injury through their own FcγRs. Further, our data indicate that each of the FcγRs specializes in separate steps leading to organ injury.

RESULTS

Generation of Mice with Neutrophil-Selective Expression of Human FcγRIIA and FcγRIIIB and Analysis of Receptor Expression

Human neutrophil FcγRs were placed under the control of the human MRP8 promoter (Figure S1 available online), which drives expression primarily in the myeloid lineage (Lagasse and Weissman, 1994). The two human FcγR transgenics, and a third line generated by breeding mice expressing the single transgenes, were bred to Fcγ-chain-deficient (*Fcer1g*$^{-/-}$) mice to produce animals that express the activating human FcγRIIA and/or FcγRIIIB on neutrophils in the absence of endogenous murine activating FcγRs. These transgenic lines are referred to as IIA^{+}*Fcer1g*$^{-/-}$, IIIB^{+}*Fcer1g*$^{-/-}$, and IIA,IIIB^{+}*Fcer1g*$^{-/-}$. Flow-cytometric analysis revealed that the human proteins were present on greater than 85%–95% of peripheral-blood transgenic neutrophils and on a population of monocytes (FcγRIIA on 20% and FcγRIIIB on 70% of cells) (Figure 1A). Both receptors were largely absent on macrophages, mast cells, CD3^{+} T cells, platelets (Figure 1B), and B cells (data not shown). PMA stimulation resulted in FcγRIIIB shedding from the surface of activated transgenic neutrophils (Figure 1C) as reported for human neutrophils (Huizinga et al., 1988), indicating similar regulation of the receptor in transgenic murine and human cells.

The relative expression of the human transgenic versus endogenous mouse FcγRs was determined by quantitation of mRNA expression in neutrophils expressing FcγRIIA or FcγRIIIB on a wild-type background. This approach, rather than the antibody-mediated detection of protein amount, was pursued because different antibodies may have differing affinities for their targets and therefore cannot be directly compared. Importantly, mRNA transcript expression of the two human FcγR transgenes and the endogenous murine FcγRIII was similar as detected by quantitative RT-PCR (Figure 1D). To determine how closely the degree of expression of human FcγRs on murine neutrophils recapitulates that observed in human neutrophils, we compared human FcγR surface expression in murine transgenic and human neutrophils by fluorescence-activated cell sorting (FACS) analysis. Transgenic FcγRIIA protein expression was equivalent, whereas FcγRIIIB on transgenic neutrophils was reduced compared to human neutrophils (Figure 1E).

FcγRIIA but Not FcγRIIIB Engagement Results in the ROS Generation

The contribution of the human FcγRs to IC-mediated adhesion and the release of ROS was evaluated. Similar numbers of isolated transgenic and wild-type neutrophils adhered to immobilized BSA-anti-BSA ICs. Despite this, the morphology and F-actin distribution in IIIB^{+}*Fcer1g*$^{-/-}$ neutrophils was altered. That is, IIA^{+}*Fcer1g*$^{-/-}$, IIA,IIIB^{+}*Fcer1g*$^{-/-}$, and wild-type neutrophils contained distinct lamellipodia enriched in cortical actin, whereas these structures were largely absent in IIIB^{+}*Fcer1g*$^{-/-}$ cells (Figure 2A). Adhesion-dependent H_2O_2 production was comparable in the wild-type, IIA^{+}*Fcer1g*$^{-/-}$, and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ but was minimal in the IIIB^{+}*Fcer1g*$^{-/-}$ and *Fcer1g*$^{-/-}$ neutrophils (Figure 2A and 2B). To bypass any effects of defective adhesion on ROS generation, we examined FcγR cross-linking-induced ROS generation in cells in suspension. FcγRIIA cross-linking on IIA^{+}*Fcer1g*$^{-/-}$ or IIA,IIIB^{+}*Fcer1g*$^{-/-}$ neutrophils resulted in robust ROS generation (Figure 2C). In contrast, FcγRIIIB cross-linking resulted in minimal ROS generation in IIIB^{+}*Fcer1g*$^{-/-}$ that was only marginally increased in IIA,IIIB^{+}*Fcer1g*$^{-/-}$ neutrophils (Figure 2C). FcγRIIA-induced ROS generation was inhibited with pharmacological inhibitors of Src, Syk kinase, and phosphatidylinositol 3-kinase (Figure 2C) and was associated with tyrosine phosphorylation

(D) Messenger RNA expression of endogenous mouse FcγRIII (open bar) and transgenic human FcγRs (filled bars) was measured by quantitative RT-PCR in mouse BMN isolated from wild-type mice (*Fcer1g*$^{+/+}$) expressing the IIA or IIIB transgene and reported relative to β-actin. The results are shown as average ± standard deviation (SD) of n = 4 per group.

(E) Comparison of FcγRs on human PMN (hPMN) and mouse BMNs (mBMN) from IIA,IIIB^{+}*Fcer1g*$^{-/-}$ animals by flow-cytometry analysis. Solid lines indicate staining for hFcγRIIA or FcγRIIIB; the mean fluorescence intensity is given. Dotted line shows the cell populations stained with isotype IgG control.

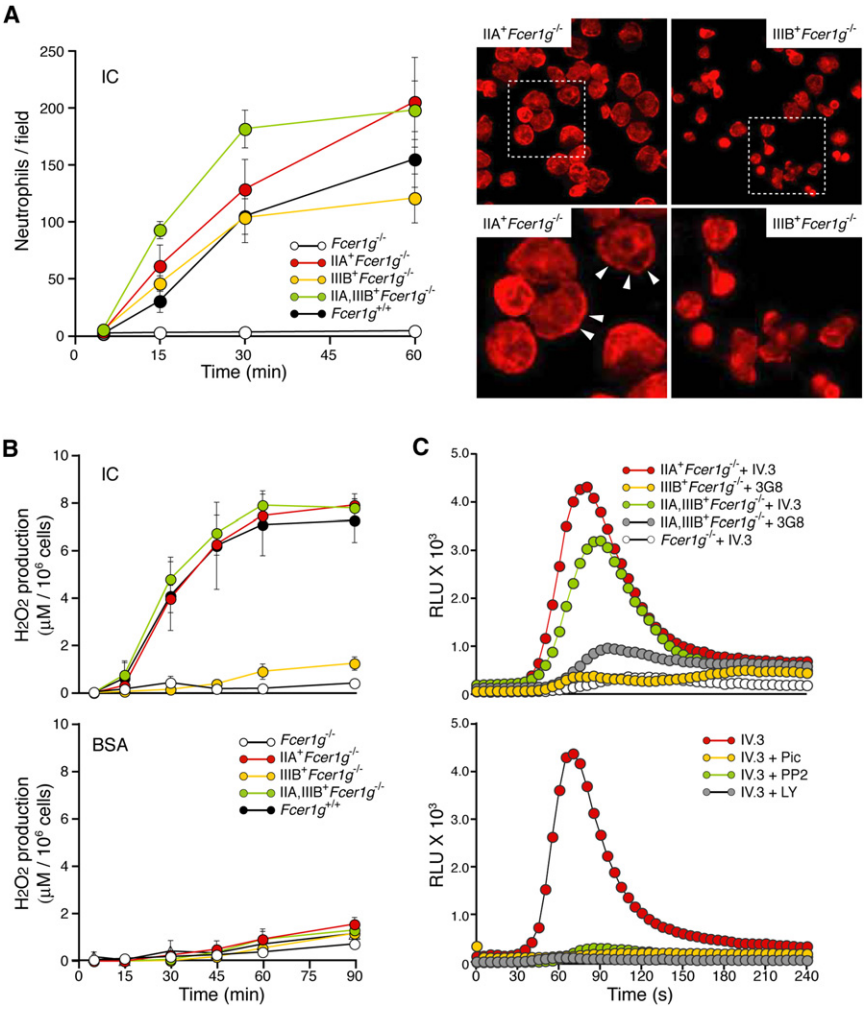

Figure 2. In Vitro Analysis of Neutrophil Adhesion to Immune Complexes and the ROS Generation

Neutrophils isolated from bone marrow of the indicated mouse strains were placed on plates coated with BSA-anti-BSA immune complexes (IC) or BSA alone. Wild-type mice are denoted as *Fcer1g*$^{+/+}$.

(A) The average number of adherent neutrophils on IC was quantitated (left). All data are mean ± standard error of the mean (SEM) of four independent experiments. Representative pictures of neutrophils adherent to immobilized IC for 30 min taken at low (top) and high (bottom) power are shown to the right. IIA^{+}*Fcer1g*$^{-/-}$ neutrophils spread with prominent cortical F-actin (arrow heads), while IIIB^{+}*Fcer1g*$^{-/-}$ neutrophils failed to do so and remained retracted.

(B) H_2O_2 concentration in culture supernatant harvested from neutrophils adherent to IC (top panel) or BSA alone (bottom panel). All data are mean ± SEM of three or four independent experiments.

(C) Cross-linking induced ROS generation. Upper panel: neutrophils from indicated mice were preincubated with mouse anti-human FcγRIIA (IV.3) or anti-FcγRIII (3G8) after GM-CSF priming. Real-time generation of ROS was monitored upon addition of F(ab')$_2$ goat anti-mouse IgG (GAM) via a luminol-based assay. The ROS profile of *Fcer1g*$^{-/-}$ neutrophils after FcγRIIA cross-linking is also shown as a control. Lower panel: real-time generation of ROS was evaluated in IIA^{+} *Fcer1g*$^{-/-}$ neutrophils treated with Piceatannol (Syk inhibitor), PP2 (Src inhibitor), or LY294002 (PI3K inhibitor), primed with GM-CSF and subjected to FcγRIIA crosslinking with IV.3. One of three representative experiments is shown.

(data not shown), as is expected for ITAM-based signal transduction (Underhill and Goodridge, 2007). Thus, FcγRIIA engages the expected ITAM-based signaling machinery in murine neutrophils to trigger ROS generation, whereas FcγRIIIB does not link to this effector response.

Human Neutrophil FcγRs Are Sufficient to Restore Progressive Glomerulonephritis in *Fcer1g*$^{-/-}$ Mice

Progressive nephrotoxic serum (NTS) nephritis in mice is a prototypic type II-hypersensitivity response in the kidney induced by antibody directed against the glomerular basement membrane (GBM). Presensitization of mice with rabbit IgG prior to challenge with rabbit NTS results in glomerular injury and renal dysfunction that resembles aspects of Goodpasture syndrome in humans (Neale and Wilson, 1982). NTS-induced renal damage relies on heterologous (rabbit or sheep) rather than autologous mouse IgG (Dean et al., 2005; Li et al., 1997; Rosenkranz et al., 1999). Whereas *Fcer1g*$^{-/-}$ mice subjected to progressive NTS nephritis failed to develop disease (Figures 3A–3E) as reported (Park et al., 1998), IIA^{+}*Fcer1g*$^{-/-}$ exhibited mortality, renal dysfunction (elevation of urine albumin and serum creatinine), and histopathologic evidence of significant glomerular and interstitial damage (Figures 3A–3E). Renal injury was absent in IIIB^{+}*Fcer1g*$^{-/-}$ mice (Figures 3A–3E). However, mice expressing both FcγRIIA and FcγRIIIB exhibited considerably more disease compared to animals expressing FcγRIIA alone, indicating cooperation between FcγRIIA and FcγRIIIB. Compared to wild-type animals, mice expressing IIA, or IIA and IIIB, had earlier onset of disease, greater disease severity, and ultimately mortality (Figures 3A–3E). These data suggest a primacy of human neutrophil FcγRs in glomerular inflammation and pathology.

NTS nephritis is associated with glomerular inflammatory cell infiltration as a result of antibody deposition (Fries et al., 1988) and secondary interstitial leukocyte accumulation as a consequence of damage to the glomerulus (Anders et al., 2003). Glomerular neutrophil accumulation was minimal in *Fcer1g*$^{-/-}$ mice (Figure 4A), as previously shown (Suzuki et al., 1998). In contrast, a striking increase in neutrophil influx was observed in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ at day 7 after disease induction (Figure 4A). The apparent decrease at later time points probably reflects the destruction or clearance of neutrophils in the inflamed tissue. Substantial glomerular neutrophil accumulation was also detected in IIIB^{+}*Fcer1g*$^{-/-}$ mice. However, this was not accompanied by renal injury in these animals (Figure 3),

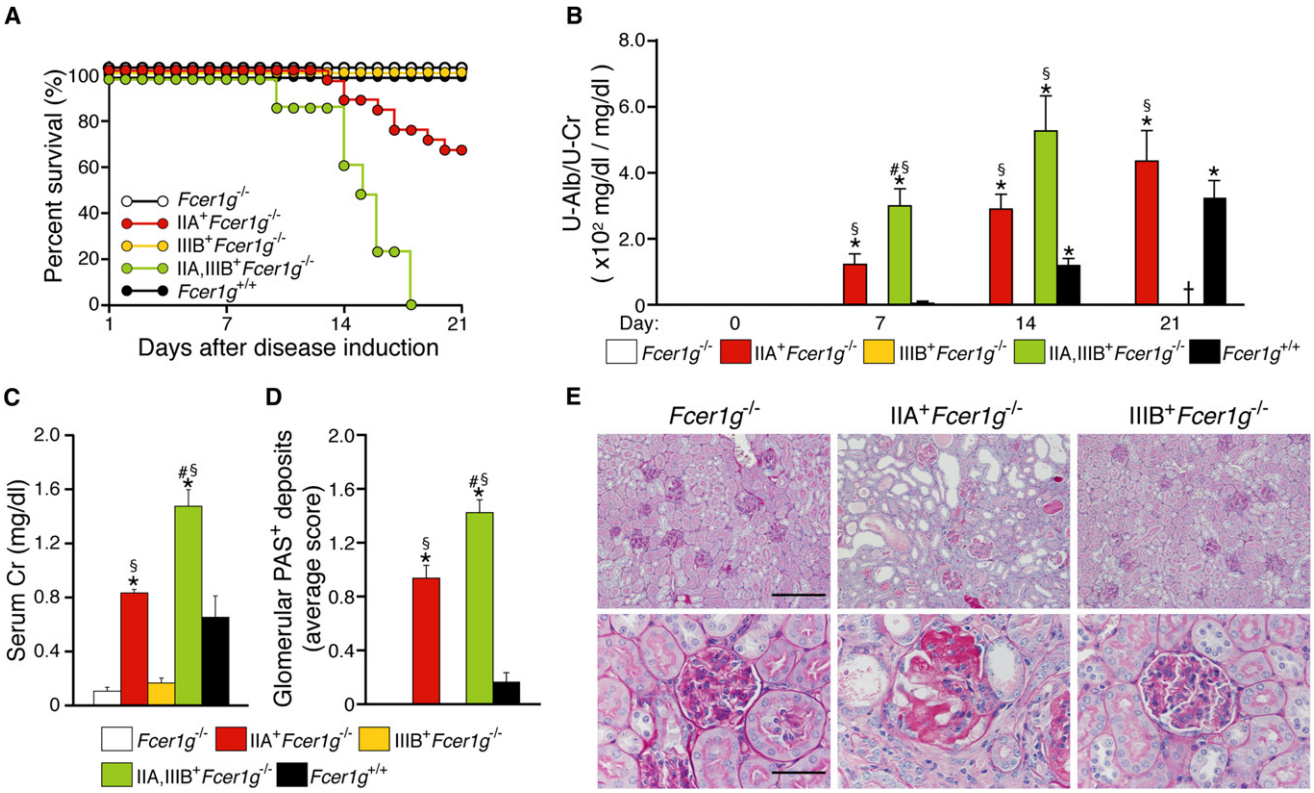

Figure 3. Analysis of Progressive NTS Nephritis

Mice were preimmunized with rabbit IgG in incomplete Freud's adjuvant on day −3 and injected intravenously with NTS on day 0. Wild-type mice are denoted as *Fcer1g*$^{+/+}$.

(A) Survival of five different strains of mice after induction of disease. IIA^{+}*Fcer1g*$^{-/-}$ mice (n = 23) and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice (n = 8) showed high mortality. All IIIB^{+}*Fcer1g*$^{-/-}$, *Fcer1g*$^{-/-}$, and *Fcer1g*$^{+/+}$ mice (n = 12–16 per group) survived the entire experimental time period.

(B and C) Analysis of renal function. Albuminuria (n = 8–23 per group) (B) and serum creatinine at day 14 (n = 6–11 for each group) (C) were elevated in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice compared to *Fcer1g*$^{-/-}$ and IIIB^{+}*Fcer1g*$^{-/-}$, and disease was accelerated in IIA^{+}*Fcer1g*$^{-/-}$, IIA,IIIB^{+}*Fcer1g*$^{-/-}$ compared to wild-type mice.

(D) A quantitation of glomerular PAS deposits of indicated strains at day 7 is shown (n = 6–10 per group). All data are mean ± SEM $p < 0.001$ by ANOVA among all strains of mice.

(E) Representative pictures of periodic acid-schiff (PAS)-stained sections of kidney harvested on day 21. Deposition of PAS-positive material (indicative of glomerular damage), occlusion of the glomerular capillary lumen, and adhesion to Bowman's capsule, as well as interstitial damage including tubular dilation, severe tubular cell atrophy, and cast formation, were observed only in IIA$^{+}\gamma^{-/-}$ mice. The bars represent 200 μm (upper) and 50 μm (lower).

Tukey-Kramer was done for comparison between two mouse strains at 5% significant level. *, versus *Fcer1g*$^{-/-}$ and IIIB^{+}*Fcer1g*$^{-/-}$; #, versus IIA^{+}*Fcer1g*$^{-/-}$; §, versus C57Bl/6.

implying that the recruited neutrophils are not activated. Elevated glomerular neutrophil counts were observed in IIA,IIIB^{+}*Fcer1g*$^{-/-}$ compared to mice expressing either transgene alone, and this far exceeded that observed in wild-type mice (Figure 4A). In the renal interstitium, infiltration of neutrophils, macrophages, and CD4^{+} T cells was observed in both IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice (Figures 4B and 4C) and correlated with the severity of glomerular injury observed (see Figure 3). Interstitial leukocytic infiltration was minimal in IIIB^{+}*Fcer1g*$^{-/-}$ (Figure 4B), which correlates with a lack of glomerular injury in this group of animals (Figures 3B–3E). CD3^{+} T cells and F4/80^{+} macrophages in renal infiltrates remained negative for hFcγR expression (data not shown).

Together, the data demonstrate that human neutrophil FcγR expression is sufficient to restore progressive glomerulonephritis in *Fcer1g*$^{-/-}$ mice. Both human neutrophil FcγRIIA and the GPI-linked FcγRIIIB promote glomerular neutrophil accumulation in response to in situ-formed glomerular ICs and cooperate in this activity. However, FcγRIIA alone is responsible for glomerular injury that results in interstititial macrophage, neutrophil and T cell accumulation, and associated renal dysfunction. The enhanced phenotypes in the IIA,IIIB^{+}*Fcer1g*$^{-/-}$ versus wild-type mice suggest that the human and murine FcγRs are not functionally equivalent.

Human Neutrophil FcγRs Are Sufficient to Restore the RPA Reaction in *Fcer1g*$^{-/-}$ Mice

Mice were subjected to the cutaneous RPA reaction that is elicited by soluble ICs. Edema and neutrophil accumulation were reduced in *Fcer1g*$^{-/-}$ mice compared to wild-type mice (Figures 5A and 5B and Figure S2), as previously reported (Sylvestre and Ravetch, 1994). In contrast, robust edema was observed in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice and was associated with significant dermal neutrophil accumulation (Figures 5A

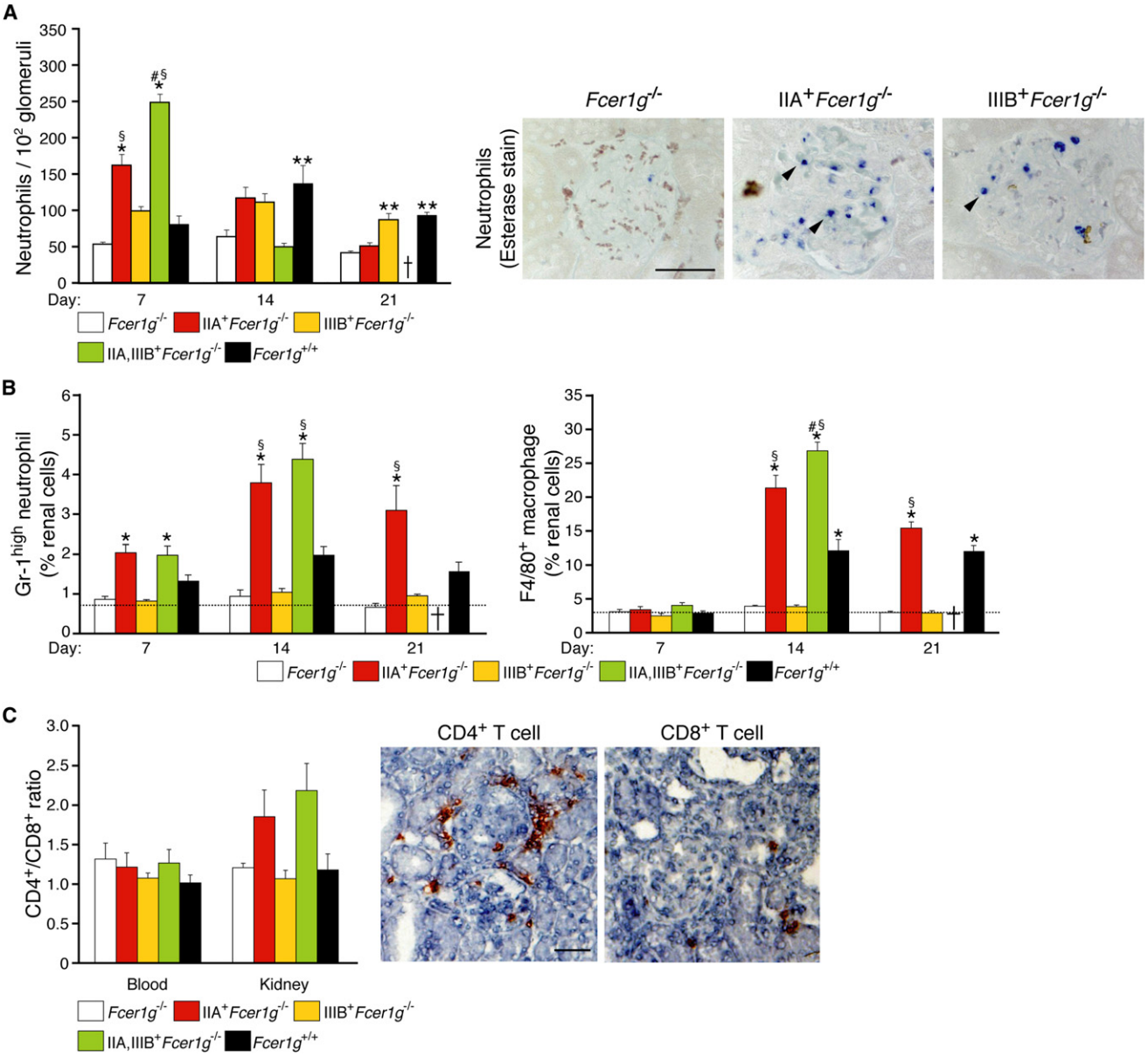

Figure 4. Analysis of Renal Neutrophil and Macrophage Accumulation in Progressive NTS Nephritis

Wild-type mice are denoted as *Fcer1g*$^{+/+}$.

(A) Glomerular neutrophil accumulation was evaluated at the indicated time points (n = 5–14 per group) after induction of progressive NTS nephritis. Representative pictures of sections with neutrophil-specific esterase stain are shown from day 7 samples. Arrowheads indicate esterase-positive neutrophils. The bar represents 50 μm.

(B and C) Analysis of interstitial infiltrates of kidneys harvested at the indicated days after NTS injection by FACS analysis of renal single-cell suspensions. Percentages of interstitial neutrophils (Gr-1^{hi}F4/80^{-}) and monocytes and macrophages (F4/80^{+}) (B) are given. Dotted line indicates baseline levels of infiltrates present in nontreated *Fcer1g*$^{-/-}$ mice. In (C), the ratio of CD4^{+} to CD8^{+} T cells in renal cell infiltrates and peripheral blood is given. Representative renal sections from IIA^{+}*Fcer1g*$^{-/-}$ mice immunohistochemically stained for CD4^{+} and CD8^{+} T cells revealed significant periglomerular infiltration of CD4^{+} T cells, whereas CD8^{+} T cells were primarily restricted to the interstitium. The bar represents 50 μm. n = 7–14 animals per group. All data are mean ± SEM. Statistics are as described in Figure 3.

and 5B and Figure S2). Mice expressing both human FcγRs exhibited disease indices in excess of wild-type mice. In contrast, edema was largely absent in IIIB^{+}*Fcer1g*$^{-/-}$ mice despite neutrophil accumulation that was comparable to that seen in wild-type animals (Figures 5A and 5B and Figure S2). Thus, human neutrophil FcγR expression in *Fcer1g*$^{-/-}$ mice was sufficient to support the RPA reaction. FcγRIIA and FcγRIIIB mediated neutrophil accumulation despite the absence of FcγR expression in resident mast cells, and FcγRIIA elicited tissue injury and together with FcγRIIIB promoted a RPA reaction that was in excess of that observed in wild-type animals expressing endogenous murine FcγRs.

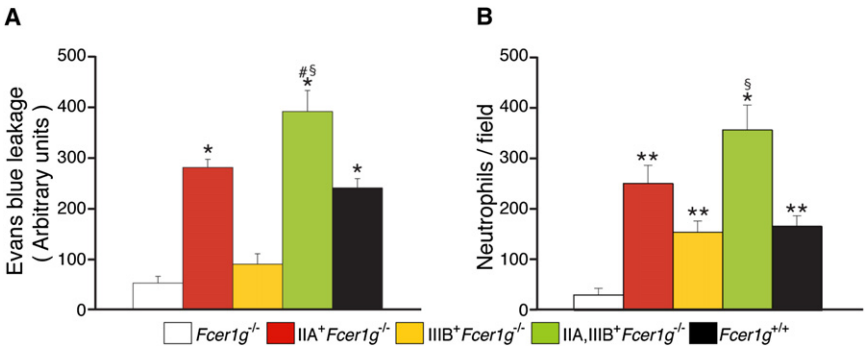

Figure 5. Analysis of the Reverse Passive Arthus Reaction

Wild-type mice are denoted as *Fcer1g*$^{+/+}$. The reverse passive Arthus reaction was induced by the intradermal administration of anti-OVA antibody and the intravenous injection of OVA with or without Evans blue dye.

(A) Evans blue in skin was quantitated by blue-dye extraction in dimethyformamide and measurement of absorbance at OD_{595}.

(B) Neutrophil accumulation in mice not given Evans blue was assessed on esterase-stained skin sections harvested 4 hr after disease induction. Average cell number ± SEM is given. n = 8–11 mice per group. Statistics are as described in Figure 3.

Evidence for Human Neutrophil FcγRIIA and FcγRIIIB Tethering to Immune Complexes Formed In Situ

A widely held view of antibody-mediated disease is that FcγR-bearing resident tissue cells promote inflammatory cell infiltration. Our results indicate that neutrophil FcγRs, in the absence of FcγR-expressing mast cells and macrophages, support neutrophil recruitment after induction of progressive NTS glomerulonephritis and the cutaneous RPA. To explore whether direct tethering of neutrophil FcγRs to ICs deposited in the vessel wall promotes neutrophil accumulation, we evaluated the mice in an acute model of NTS. As with the progressive NTS model, neutrophil accumulation occurs as a result of antibody binding to the glomerular basement membrane that is exposed to circulating cells through open fenestrae (Fries et al., 1988; Reeves et al., 1980). However, in the acute model, NTS delivered in the absence of IgG presensitization results in rapid, transient glomerular neutrophil influx. The relative absence of tissue injury in the acute model minimizes secondary effects in neutrophil accumulation. Glomerular neutrophil influx was minimal in *Fcer1g*$^{-/-}$ mice (Figure 6), as reported (Coxon et al., 2001; Suzuki et al., 2003). In contrast, neutrophil recruitment that was observed in IIIB^{+}*Fcer1g*$^{-/-}$ and wild-type mice peaked at 1 hr after NTS injection. Neutrophil accumulation in IIA^{+}*Fcer1g*$^{-/-}$ was also prominent at the 1 hr time point, but this was sustained and enhanced compared to wild-type and IIIB^{+}*Fcer1g*$^{-/-}$ animals at time points up to 4 hr after NTS injection. This response was further increased in IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice compared to either of the single transgenics (Figure 6). These data suggest that neutrophil accumulation is supported by neutrophil FcγRIIA and FcγRIIIB tethering to in situ-formed glomerular ICs.

Differential Contributions of Human Neutrophil FcγRs in Slow Rolling, Adhesion, and Transmigration in Models of Soluble-IC Deposition

Here we examined the contribution of human FcγRs to neutrophil recruitment in response to soluble ICs. For this, two independent models of soluble-IC deposition amenable to intravital microscopy were exploited. In the first model, the RPA was induced in the cremaster muscle by the intrascrotal injection of anti-ovalbumin (OVA) and the intravenous delivery of OVA. Neutrophil recruitment in this model requires complement and TNF as well as cellular responses of mast cells, platelets, and activated endothelial cells (Lister et al., 2007; Norman et al., 2005; Norman et al., 2003). When applied to the skin, the RPA results in IC deposition within the vessel and in the perivascular and extravascular space (Cream et al., 1971; Jancar and Sanchez Crespo, 2005). The RPA did not increase the rolling-flux fraction in wild-type mice (data not shown) but did induce significantly slower leukocyte rolling velocities and enhanced adhesion and transmigration compared to animals treated with OVA alone (Figures 7A–7C). The rolling-flux fraction was similar between wild-type, transgenic, and *Fcer1g*$^{-/-}$ mice (data not shown). However, leukocyte rolling velocities were reduced in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice compared to wild-type and *Fcer1g*$^{-/-}$ animals, suggesting that human FcγRIIA promotes slow rolling (Figure 7A). The transition to slow rolling in other models of inflammation is associated with firm arrest (Ley et al., 2007). Consistent with this, slow rolling was a reliable predictor of IC-mediated adhesion because the latter was elevated in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice compared to *Fcer1g*$^{-/-}$ animals (Figure 7B). Transmigration, which is a time-dependent variable of adhesion, was also elevated in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ mice compared to

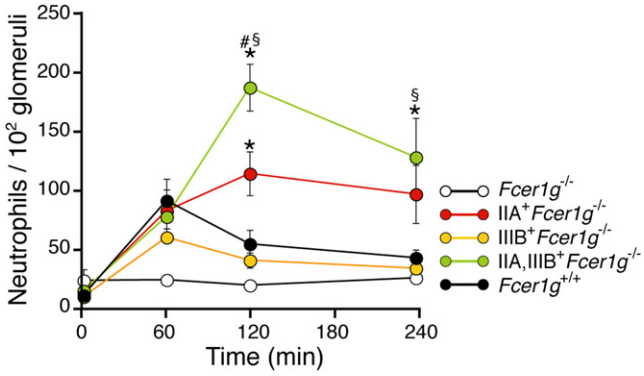

Figure 6. Analysis of Neutrophil Influx after Acute NTS Nephritis

Mice were injected with NTS, kidneys were harvested at the indicated time points, and neutrophil accumulation was enumerated in neutrophil-specific esterase-stained tissue sections. Wild-type mice are denoted as *Fcer1g*$^{+/+}$. Comparable neutrophil recruitment was observed in wild-type mice and all three transgenic animals at the 1 hr time point. At later time points, neutrophil accumulation was further increased in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ but declined in IIIB^{+}*Fcer1g*$^{-/-}$ and wild-type mice. n = 6–12 per group. All data are mean ± SEM. Statistics are as described in Figure 3.

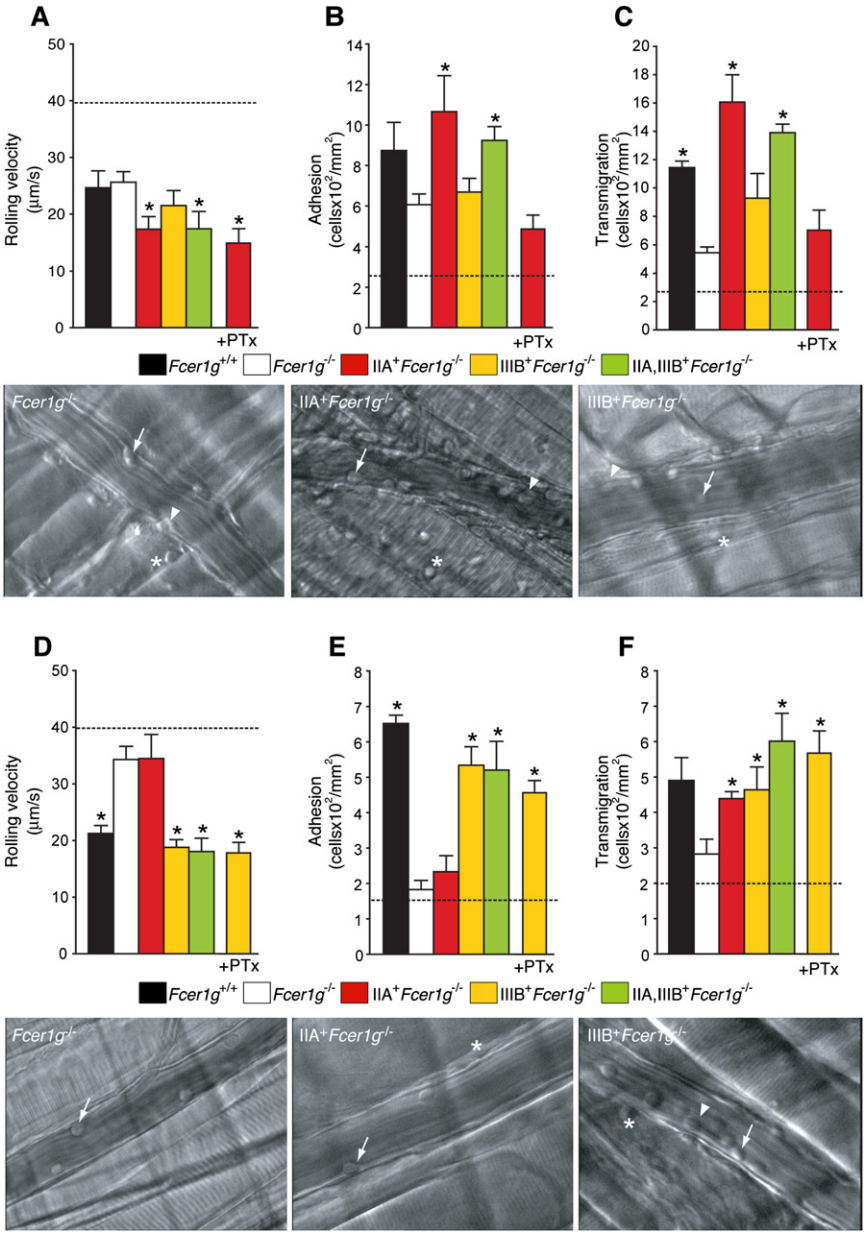

Figure 7. Intravital Microscopic Analysis of Neutrophil Recruitment after Deposition of Soluble ICs

In panels (A)–(C), the RPA was induced in the cremaster of the indicated mice by an intrascrotal injection of anti-OVA followed by an intravenous injection with OVA. Wild-type mice are *Fcer1g*$^{+/+}$. Leukocyte rolling velocity (A), adhesion (B), and transmigration (C) were evaluated in each group. The same was evaluated in wild-type mice (*Fcer1g*$^{+/+}$) after injection with OVA alone, and the average is presented as a dashed line. IIA^{+}*Fcer1g*$^{-/-}$ mice pretreated with pertussis toxin (+PTx) are indicated. After IC injection, IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ exhibited slow rolling, whereas IIIB^{+}*Fcer1g*$^{-/-}$ failed to do so (A). Compared to *Fcer1g*$^{-/-}$, adhesion (B) and transmigration (C) were significantly increased in IIA^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$ but not in IIIB^{+}*Fcer1g*$^{-/-}$. Representative pictures of a postcapillary venule from *Fcer1g*$^{-/-}$, IIA^{+}*Fcer1g*$^{-/-}$, and IIIB^{+}*Fcer1g*$^{-/-}$ mice with rolling (arrow), adherent (arrowhead), and transmigrated (asterisk) neutrophils are shown. Pertussis toxin pretreatment (PTx) completely suppressed the adhesion and transmigration of leukocytes in IIA^{+}*Fcer1g*$^{-/-}$.

In panels (D)–(F), the indicated mice were injected intravenously with preformed soluble BSA-anti-BSA ICs, and the cremaster was exteriorized for intravital microscopy. IIIB^{+}*Fcer1g*$^{-/-}$ mice given pertussis toxin (+PTx) are indicated. Leukocyte rolling velocity (D), adhesion (E), and transmigration (F) were evaluated in each group. The same was evaluated in wild-type mice after injection with BSA alone, and the average is presented as a dashed line. Slow leukocyte rolling (D) and adhesion (E) were observed in *Fcer1g*$^{+/+}$ mice injected with ICs compared to mice given BSA alone, whereas this behavior was absent in *Fcer1g*$^{-/-}$ mice. After IC injection, IIIB^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$, but not IIA^{+}*Fcer1g*$^{-/-}$, exhibited significant slow rolling (D) and adhesion (E). Transmigration was prominent in all groups of animals except *Fcer1g*$^{-/-}$ mice (F). PTx had no effect on slow rolling, adhesion, and transmigration in IIIB^{+}*Fcer1g*$^{-/-}$ mice. Representative pictures of neutrophils interacting with postcapillary venules from *Fcer1g*$^{-/-}$, IIA^{+}*Fcer1g*$^{-/-}$, and IIIB^{+}*Fcer1g*$^{-/-}$ mice are shown.

All data are mean ± SEM. *, $p < 0.05$, $n = 5$ per group.

Fcer1g$^{-/-}$ mice (Figure 7C). G protein-coupled receptors (GPCRs) play major roles in leukocyte interactions with the vessel wall in non-IC-mediated inflammation models (Ley et al., 2007). An intravenous injection of pertussis toxin, an inhibitor of Gα_i-coupled receptors, prior to the induction of the RPA was effective as assessed by the inhibition of fmlp-mediated ROS generation in peripheral-blood neutrophils sampled at the end of the intravital microscopy procedure (data not shown). Under these treatment conditions, pertussis toxin completely abrogated FcγRIIA-mediated adhesion and transmigration (Figures 7B and 7C) but had no effect on slow rolling (Figure 7A), rolling-flux fractions, or peripheral-blood leukocyte counts (data not shown). In contrast to FcγRIIA, FcγRIIIB failed to significantly support neutrophil recruitment because slow rolling and adhesion was not observed in IIIB^{+}*Fcer1g*$^{-/-}$ mice (Figures 7A and 7B). Thus, in this model FcγRIIA cooperates with Gα_i-coupled receptors to enhance soluble IC-induced leukocyte adhesion and transmigration, whereas FcγRIIIB does not significantly participate in this process.

Next, mice were evaluated after the deposition of preformed anti-BSA and BSA soluble ICs. In this model, intravenously delivered ICs rapidly deposit within the vessel wall as a result of changes in vascular permeability induced by cremaster exteriorization (Stokol et al., 2004). IC deposition promotes neutrophil slow rolling on P selectin and adhesion and transmigration that are dependent on murine activating FcγRs (Stokol et al., 2004). Rolling-flux fractions did not increase in mice given preformed ICs compared to mice given BSA alone as described (Stokol

et al., 2004), and rolling-flux fractions were similar between all animal groups (data not shown). ICs induced slow rolling, adhesion, and transmigration in wild-type mice that were significantly attenuated in *Fcer1g*$^{-/-}$ mice (Figures 7D–7F), as reported (Stokol et al., 2004). However, in contrast to the cremaster RPA, FcγRIIIB was solely required for slow rolling, adhesion, and transmigration in this model because only IIIB^{+}*Fcer1g*$^{-/-}$ and IIA,IIIB^{+}*Fcer1g*$^{-/-}$, but not IIA^{+}*Fcer1g*$^{-/-}$, supported these processes compared to *Fcer1g*$^{-/-}$ animals (Figures 7D–7F, and see Movies S1–S3). IIA^{+}*Fcer1g*$^{-/-}$ mice had measurable numbers of extravascular neutrophils (Figure 7F), suggesting that the small number of adherent FcγRIIA-expressing neutrophils efficiently transmigrate. For examination of the potential contribution of GPCRs to FcγRIIIB-mediated neutrophil interactions with the vessel wall, IIIB^{+}*Fcer1g*$^{-/-}$ mice were pretreated with pertussis toxin prior to the injection of preformed soluble ICs. Pertussis toxin had no effect on FcγRIIIB-mediated slow rolling, adhesion, or transmigration. Thus, under conditions of primarily intravascular ICs, and in the absence of activation of $G\alpha_i$-coupled receptors, FcγRIIIB specializes in neutrophil recruitment.

DISCUSSION

Our data demonstrate that expression of human FcγRs selectively on neutrophils is sufficient to induce virtually all aspects of type II and type III autoimmune responses and hence may provide critical molecular links between antibodies and immunological injury. Neutrophils were recruited via FcγRIIA and FcγRIIIB, and FcγRIIA alone signaled tissue injury. The individual human neutrophil FcγRs appear to play separate roles in IC-induced neutrophil recruitment in response to both ICs formed in situ and soluble ICs deposited in the vessel wall. In the case of soluble ICs, FcγRIIIB specialized in neutrophil interactions in the context of strictly intravascular ICs, whereas FcγRIIA predominated in the RPA reaction, which is a more complex response induced by vascular and tissue ICs and GPCR-binding chemokines. In the case of in situ-generated ICs, FcγRIIIB and FcγRIIA initiated recruitment while FcγRIIA was required for sustained neutrophil accumulation. Neutrophil recruitment and organ injury in the transgenic line expressing the human complement of neutrophil FcγRs (FcγRIIA and FcγRIIIB) far exceeded that observed in wild-type mice (FcγRIII and FcγRIV) despite equivalent expression of the human and murine activating receptors. This provides evidence that the human and mouse neutrophil FcγRs are not functionally equivalent and in humans may play a primary role in initiating IC-mediated diseases.

The greater tissue damage observed in mice expressing the human FcγRs compared to the murine FcγRs (i.e., wild-type mice) may be the result of intrinsic differences such as more effective ITAM-based signal transduction leading to neutrophil cytotoxicity (Van den Herik-Oudijk et al., 1995) or a differential capacity of the human and murine FcγRs to support neutrophil recruitment to ICs under conditions of limiting amounts of ICs in vivo. However, we cannot rule out the possibility that extrinsic factors, such as the relative degree of interactions between the activating FcγRs with murine FcγRIIB, and/or a potential downregulatory role for murine FcγRs in other cell types accounts for these differences. Implicit in our results is the possibility that the contribution of neutrophil-expressed murine FcγRs may have been underestimated in past studies. This may indeed be the case because mast cells and FcγRs on macrophages are only partially responsible for the RPA reaction and progressive NTS nephritis, respectively (Bergtold et al., 2006; Sylvestre and Ravetch, 1996).

Our studies suggest that the fundamental assumptions of the pathogenesis of hypersensitivity disease may require re-evaluation in the case of human inflammation. Our finding that neutrophils are sufficient to promote type II and type III hypersensitivity requires modification of the current paradigm primarily deduced from studies in knock-out mice, which suggest that FcγR-expressing tissue-resident cells initiate IC-mediated inflammatory reactions (Schmidt and Gessner, 2005). Our finding that human FcγRs on neutrophils play a primary role in progressive NTS nephritis suggests the possibility that neutrophils, and in particular FcγRs on neutrophils, may play a dominant role in the pathogenesis of IC-mediated glomerulonephritides. This suggests a broader significance for neutrophils in these conditions than previously anticipated. Importantly, neutrophils have been documented in renal biopsies from patients with membranoproliferative, lupus, and crescentic glomerulonephritis (Camussi et al., 1980; Hooke et al., 1987; Segerer et al., 2006). Human FcγRs were also observed in a subpopulation of monocytes in our transgenic lines. Therefore, FcγRs on monocytes may be required for the full expression of renal disease. However, it is noteworthy that *Fcer1g*$^{-/-}$ mice with transgenic re-expression of the γ-chain and thus activating FcγRs in monocytes and macrophages, with no detectable expression in neutrophils, continued to exhibit a 70% decrease in indices of glomerular damage compared to wild-type counterparts (Bergtold et al., 2006). This argues that FcγR expression in monocytes is not sufficient for disease induction. Glomerular-injury-associated proteinuria itself, as well as chemokines secreted by the glomerulus, stimulates tubular epithelial cells to secrete chemokines that support interstitial leukocyte infiltration (Anders et al., 2003). Our data indicate that FcγRIIA-mediated glomerular neutrophil recruitment and proteinuria promote subsequent interstitial influx of macrophage and T cells, which are effector populations known to contribute to disease pathogenesis and end-stage renal failure (Duffield et al., 2005; Hooke et al., 1987; Tipping and Holdsworth, 2006).

FcγRIIA alone was sufficient to mediate immunological injury in vivo. The demonstration that reconstitution with an FcγR is critical in disease models shown previously to be dependent on the γ-chain is noteworthy because this ITAM-based adaptor is also central to the regulation of receptors important in MHC-I recognition and myeloid cell and platelet activation, raising the possibility that some of the phenotypes in *Fcer1g*$^{-/-}$ mice may be attributed to deficiency in signaling through these receptors (Fodor et al., 2006; Mocsai et al., 2006; Underhill and Goodridge, 2007). The differential cytotoxic activity of the ITAM-containing FcγRIIA versus the GPI-linked FcγRIIIB is probably related to a requirement for ITAM-based signal transduction in the generation of neutrophil effector functions such as ROS generation, as demonstrated by our in vitro studies of transgenic neutrophils and published reports on human neutrophils (Underhill and Goodridge, 2007). Furthermore, overexpression of ITAM-containing human FcγRIIA or FcαRI on monocytes and macrophages in wild-type mice has been reported to aggravate arthritis and glomerulonephritis development (Kanamaru et al., 2007;

Tan Sardjono et al., 2005). On the other hand, FcγRIIIB may fail to exhibit cytotoxicity in vivo relative to FcγRIIA because it is shed from the surface of neutrophils accumulated in the inflamed tissue.

An important finding was that expression of human FcγRs on neutrophils was sufficient to elicit neutrophil recruitment. This is a complex process previously attributed to endothelial cell-adhesion molecule (CAM) upregulation by cytokines released from activated endothelial cells and/or by other cells within the vessel wall to which antibody is bound (Nikolic-Paterson et al., 1994; Norman et al., 2003; Schmidt and Gessner, 2005). The human FcγR transgenics exhibited significant neutrophil accumulation in tissues despite the absence of these receptors on mast cells and macrophages, which indicates a primary role for the FcγRs on neutrophils in IC-induced neutrophil recruitment. FcγRIIA and FcγRIIIB played equivalent roles in initial neutrophil recruitment in response to antibody-antigen complexes within the glomerular capillaries. Given that the ICs are accessible to circulating neutrophils through open endothelial fenestrae (Fries et al., 1988), our results strongly suggest that neutrophil FcγRs directly tether to ICs. The enhanced accumulation in mice expressing FcγRIIA observed at later time points suggests an additional role for this receptor in sustaining neutrophil accumulation, perhaps by signaling the release of leukotrienes and prostaglandins (Jancar and Sanchez Crespo, 2005). Analysis of mice in intravital models of soluble-IC deposition indicated nonredundant functions for the two human FcγRs in neutrophil recruitment. FcγRIIIB was specialized for slow rolling and adhesion induced by intravascular soluble ICs in a model where neutrophil accumulation is independent of complement C3 and C5, does not require the function of mast cells, and is not associated with platelet accumulation or expression of cytokine-inducible endothelial CAMs (Stokol et al., 2004). However, in a more complex environment of intravascular, perivascular, and tissue ICs generated by the RPA (Cochrane, 1963; Cream and Turk, 1971; Movat and Fernando, 1963), FcγRIIA predominated. FcγRIIA-mediated adhesion and transmigration were dependent on $G\alpha_i$-coupled receptors. We postulate that engagement of GPCR(s) on neutrophils and/or other cell types may directly or indirectly modulate FcγRIIA-mediated adhesion by affecting its affinity for deposited IgG (Nagarajan et al., 2000) or upregulating neutrophil CD18 integrins known to support FcγR function (Jones and Brown, 1996).

The assignment of an important physiological role for FcγRIIIB in neutrophil recruitment is significant particularly because the function of this GPI-anchored receptor remains largely unknown. Furthermore, our results ascribe a function to FcγRIIIB that doesn't require FcγRIIA, a finding unanticipated from previous work (Unkeless et al., 1995). What is the physiological role of FcγRIIIB in neutrophil tethering to intravascular ICs? We propose that FcγRIIIB may clear ICs from the glomerulus, which is a frequent site of IC trapping, and thus aids in the maintenance of homeostasis (Nangaku and Couser, 2005). Indeed, glomerular ICs can trigger a transient accumulation of neutrophils with their return to the circulation and a complete clearance of the immune deposits and restoration of the glomerular structural integrity within 24 hr of IC deposition (Fries et al., 1988). The high density of FcγRIIIB with a GPI anchor nearly the size of an Ig domain that could permit it to protrude further than FcγRIIA, its predicted fast mobility in the membrane bilayer (Selvaraj et al., 1988), and its presence on microvilli (Coxon et al., 2001) coupled with its weak signaling capacity may suit FcγRIIIB for efficient capture and internalization of ICs with minimal neutrophil activation. The higher expression of FcγRIIIB in human versus our murine transgenic neutrophils predicts a more significant role for this receptor in IC-induced neutrophil accumulation in humans. FcγRIIIB is normally present in multiple gene copies that usually positively correlate with its expression, and a low gene copy number of FcγRIIIB predisposes to glomerulonephritis in systemic lupus erythematosus (Aitman et al., 2006; Stranger et al., 2007). We speculate that inefficient FcγRIIIB-mediated recruitment and clearance of ICs under homeostatic conditions may enhance susceptibility to IC-mediated diseases in patients. Pathogenic-IC accumulation may increase the local generation of inflammatory mediators that promote FcγRIIIB shedding, increase the capacity of FcγRIIA to bind ligand and signal, and thus promote tissue damage.

In summary, our studies in FcγR humanized mouse models have provided evidence that human FcγR expression on neutrophils is sufficient for the initiation of IC-induced inflammatory disease and thus redefines the currently accepted models of type II and III autoimmune responses developed in knockout mice. Neutrophils are recruited via their own FcγRs to ICs with FcγRIIA and FcγRIIIB playing distinct context-dependent roles in this process, whereas FcγRIIA alone is responsible for tissue injury. We anticipate that transgenic mice expressing the human repertoire of neutrophil FcγRs will continue to serve as important tools for investigating the physiological function of the individual receptors, their signaling capacity, and the mechanisms by which they promote tissue injury in inflammatory and autoimmune disorders relevant to human disease.

EXPERIMENTAL PROCEDURES

Generation of Human FcγR Transgenic Mice

The 0.95 kb of human FcγRIIA cDNA and 0.7 kb of FcγRIIIB cDNA were subcloned into the BglII site of the hMRP8 promoter (Lagasse and Weissman, 1994). Natural polymorphisms in FcγRIIA and IIIB have been reported. Of these, cDNAs encoding FcγRIIIB-NA2 and FcγRIIA-R131 were utilized to generate the transgenics. FcγRIIA-R131 is a natural variant of FcγRIIA in the human population that is highly responsive to mouse IgG (van der Pol and van de Winkel, 1998). Each transgene was released from pUC18-hMRP8 vector by digestion with HindIII and EcoRI and injected into zygotes from C57Bl/6J mice. Transgenic mice were generated in the transgenic facility of Brigham & Women's Hospital (Boston, MA). A high-expressing founder transgenic line of FcγRIIA transgenic mice (IIA^+) was crossed with $Fcer1g^{-/-}$ mice on a C57Bl/6J-F12 background and bred to be hemizygous for the FcγRIIA transgene and γ-chain deficient, as assessed by PCR of genomic DNA and flow-cytometry analysis. $IIIB^+Fcer1g^{-/-}$ mice were similarly generated. Mice expressing both FcγRIIA and FcγRIIIB ($IIA,IIIB^+Fcer1g^{-/-}$) were generated by breeding $IIA^+Fcer1g^{-/-}$ and $IIIB^+Fcer1g^{-/-}$ mice. Mice were maintained in a virus- and antibody-free facility at the New Research Building animal housing facility at Harvard Medical School. Mice used for each experiment were between 6 and 8 weeks of age and sex matched. The Harvard Medical School Animal Care and Use Committee approved all procedures in this study.

Isolation and Treatment of Leukocyte Populations

Peripheral blood sampled from the retro-orbital plexus of mice was collected in ethylene diamine tetra-acetic acid (EDTA)-containing tubes. Human peripheral neutrophils and murine bone-marrow neutrophils were isolated as previously described (Coxon et al., 2001; Hirahashi et al., 2006). For the assessment of FcγR shedding, mouse bone-marrow neutrophils were stimulated with or

without 100 ng/ml of PMA for 10 min at 37°C and then fixed with 4% paraformaldehyde. Bone-marrow-derived macrophages were generated by culturing cells harvested from the tibia and femurs of mice in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 20% L929-conditioned supernatant for 5 days. Peritoneal cells for FACS analysis were harvested by lavaging the peritoneum with cold PBS and plating for 18 hr on polystyrene dishes in 10% FCS in DMEM. For peritoneal mast cells, peritoneal cells were immediately used for FACS analysis.

Flow-Cytometry Analysis

All antibodies were from BD Biosciences-PharMingen unless otherwise indicated. Human FcγRIIA or FcγIIIB expression on peripheral-blood leukocytes was characterized by using fluorescein isothiocyanate (FITC) or allophycocyanin (APC)-mouse anti-human FcγRIIA (clone FLI8.26, mouse IgG2κ) or FITC-mouse anti-human FcγRIIIB (clone 3G8, mouse IgG1). Cell populations were identified by PE-rat anti-Gr-1 (Ly-6G and Ly-6C, clone RB6-8C5) for neutrophils, PE-rat anti-CD115 (clone AFS98; eBioscience) for monocytes, PE-Cy7-hamster anti-mouse CD3ε (clone 145-2C11) for T cells, APC-rat anti-mouse CD11b (Integrin α_M chain, clone M1/70) for neutrophils and monocytes, and FITC-rat anti-mouse CD62P (RB40.34) for platelets. As a positive control for receptor shedding, APC-rat anti-mouse CD62L (clone MEL-14) was used.

For analysis of resident peritoneal and bone-marrow-derived macrophages, cells were stained with PE-mouse anti-human FcγRIIA or FcγRIII antibody amd APC-rat anti-F4/80 (clone A3-1; Caltag Laboratories). Mature mast cell populations were identified with FITC-hamster anti-mouse FcεRIa (clone MAR-1; eBioscience) and PE-rat anti-mouse c-Kit (clone 2B8) antibody. FcεRIa-negative but c-Kit-positive cells in *Fcer1g*$^{-/-}$ mice with transgenic expression of human FcγRs and in *Fcer1g*$^{-/-}$ mice were analyzed for human FcγR expression.

Infiltrating renal neutrophils, macrophages, and T cells were quantitated by three-color flow cytometry. Single-cell suspensions from individual kidneys were prepared as described (Vielhauer et al., 2003). For analysis of leukocyte surface markers, cells were incubated with PE-anti-Gr-1, APC-anti-CD3ε, or APC-anti-F4/80. The amounts of positively stained neutrophils (Gr-1^{hi}F4/80^{-}) and macrophages (F4/80^{+}) were expressed as percentage of total renal cells. Renal cell suspensions were also stained with APC-anti-CD3ε, FITC-anti-CD4 (clone RM4-5) and PE-anti-CD8a (clone 53-6.7) to determine the CD4^{+} to CD8^{+} ratio in the CD3^{+} T cell population.

Quantitative Real-Time PCR

Complementary DNAs were synthesized from total RNA of bone-marrow neutrophils via a cDNA synthesis kit (Invitrogen). RT-PCR by Taq DNA polymerase (New England Biolabs) was performed with the following primer sets: mouse FcγRIIIA (QIAGEN, QT00117803); human FcγRIIA (QIAGEN, QT00042826); human FcγRIIIB (5′-CGTGCTTGAGAAGGACAGTG-3′, 5′-CTGGCTTGAGAT GAGGCTCT-3′); and mouse β-actin (5′-CCTGAGCGCAAGTACTCTGTGT-3′, 5′-GCTGATCCACATCTGCTGGAA-3′). Each RT-PCR product was inserted into pGEMT-Easy vector (Promega) for a reference standard. Quantitative real-time PCR on cDNA samples by SYBR green RT-PCR method (Bio-Rad, Hercules, CA, USA) was conducted with the indicated primer sets. Actual transcript levels of mouse FcγRIIIA and human FcγRs genes were determined against the reference standard made by serial dilution of the pGEMT-Easy vector containing FcγR templates. The relative expression of each gene was normalized against β-actin.

Neutrophil Adhesion, F-Actin Staining, and Determination of Cellular H_2O_2 Production on IC

Experimental details for adhesion assay and F-actin staining were previously described (Tang et al., 1997). Adherent cell numbers were quantitated in three independent fields at magnification 200×, and the average cell number per field was determined. Neutrophils were plated on glass coverslips coated with ICs formed by BSA and polyclonal rabbit anti-BSA antibody (Tang et al., 1997) or BSA alone in 24-well plates. The experimental protocol for cellular H_2O_2 production was essentially as described by Werner et al., 2003 (Werner, 2003), with some modifications. For the indicated time periods 1.0×10^6 bone-marrow neutrophils from each mouse strain in 1.0 ml of assay mix solution (100 μM homovanillic acid [Sigma] in HBSS, 5 U/mL horseradish peroxidase type VI [Sigma], and 1 mM HEPES [pH 7.5]) were incubated at 37°C. The plate was then centrifuged, and cell-free supernatant was collected and further incubated at 37°C for 1 hr. The reaction was terminated by the addition of 0.1 M glycine solution. The fluorescence in supernatants was measured by a fluorometer (excitation 321 nm, emission 421 nm), and the H_2O_2 concentration in each sample was determined relative to the calibration curve made by an H_2O_2 standard.

Human FcγR Cross-Linking-Mediated Oxidative Burst in Neutrophils

Bone-marrow neutrophils were incubated with 10 μg/ml mouse anti-hFcγRIIA (clone: IV.3, StemCell Technologies) or anti-hFcγRIIIB (clone 3G8, Caltag Laboratories) on ice for 30 min. Cells (2.5×10^6/sample) were washed and then incubated with 1 μg/ml of mouse GM-CSF for 30 min followed by incubation with or without piceatannol (20 μM, Sigma), PP2 (10 μM, Calbiochem), or LY294002 (20 μM, Calbiochem) at 37°C for 30 min. FcγR cross-linking was initiated by the addition of goat anti-mouse F(ab')$_2$ (100 μg/ml, Jackson ImmunoResearch Laboratories), and ROS generation was continuously monitored with a luminol-based assay as previously described (Utomo et al., 2006).

Nephrotoxic Serum Nephritis

Experimental nephrotoxic serum (NTS) nephritis was induced in 8-week-old males as previously reported (Rosenkranz et al., 2000) with the following modifications. In brief, mice were preimmunized subcutaneously in the right footpad with 0.05 mg rabbit IgG (Jackson ImmunoResearch Laboratories) in Freund incomplete adjuvant and nonviable desiccated *Mycobacterium tuberculosis* H37Ra (Difco, Michigan). Three days later, mice were injected intravenously with 50 μl heat-inactivated, filter-sterilized nephrotoxic serum. Spot urine samples and peripheral blood were collected at indicated time points after NTS injection. Both kidneys from euthanized mice were harvested for histological analysis and flow cytometry. Acute NTS nephritis was induced in 7-week-old male mice by the intravenous injection of 300 μl of nephrotoxic serum without prior preimmunization.

Functional Assessment of Renal Injury

Urine albumin concentrations and creatinine levels in urine and serum were determined by ELISA (Rosenkranz et al., 2000) and Creatinine Assay Kit (Cayman Chemical Company), respectively. Albuminuria was expressed as mg albumin per mg urinary creatinine to standardize urine albumin excretion for glomerular filtration rate and urinary concentration.

Histological Assessment of Renal Injury, Renal Neutrophil, and T Cell Accumulation

The presence of PAS-positive deposits within glomeruli was graded semiquantitatively as previously described (Rosenkranz et al., 2000). Glomerular PMN infiltration was assessed by the chloroacetate esterase reaction as reported (Coxon et al., 2001). For each animal, glomerular neutrophil counts in more than 100 glomeruli per kidney section were made. For the histological assessment of T cell accumulation, renal cryostat sections were stained with unlabeled anti-CD4 (clone RM4-5) or anti-CD8a (clone 53-6.7).

Reverse Passive Arthus Reaction

Rabbit anti-chicken egg albumin IgG (60 μg/30 μL; Cappel, Aurora, OH) or PBS alone were injected intradermally in the left or right portion of the dorsal skin in 6- to 8-week-old female mice, followed immediately by the intravenous (i.v.) injection of chicken egg ovalbumin (400 μg/mouse; Sigma-Aldrich, St. Louis, MO). Four hours later, the skin containing the injection site was removed from euthanized mouse. In cases where edema was measured, the solution of chicken egg albumin contained 0.15% Evans blue dye (Sigma-Aldrich). Measurement of Evans blue leakage and neutrophil influx was conducted as described (Utomo et al., 2008).

Intravital Microscopy

Soluble ICs were prepared as previously described (Stokol et al., 2004). Leukocyte recruitment in cremaster muscle venules was evaluated in mice within 60 min of a single i.v. injection of ICs or BSA. For the RPA in the scrotum, rabbit IgG anti-chicken egg albumin antibody (100 μg/100 μL) was injected intrascrotally, followed by the i.v. injection of chicken egg ovalbumin (240 μg/240 μL; Sigma-Aldrich, St. Louis, MO). Leukocyte recruitment in the cremaster was

evaluated 2 hr after the injection. In some cases, mice were pretreated i.v. with 4 μg of pertussis toxin (Sigma) 4 hr prior to injection of preformed soluble ICs or induction of the RPA.

The procedures for preparation of the cremaster of anesthetized mice and subsequent intravital microscopy were essentially as described (Lauterbach et al., 2008). Four venules per mouse were analyzed over a 20 min time period. At the completion of the intravital microcopy experiment, blood was collected from the retro-orbital plexus to measure total leukocyte counts. Leukocyte rolling velocities were measured by tracking single leukocytes (10/venule) over several frames and calculating distance moved per unit time (μm/s). Adherent leukocytes were defined as cells remaining stationary for 30 s and were expressed as the number of cells/ mm^2 of venule. Transmigrated leukocytes were defined as cells outside of the venule and were expressed as the number of cells/mm^2.

Statistical Analysis

In nephrotoxic anti-GBM nephritis and RPA, data were analyzed by ANOVA among five strains—*Fcer1g*$^{-/-}$, IIA^{+}*Fcer1g*$^{-/-}$, IIIB^{+}*Fcer1g*$^{-/-}$, IIA,IIIB^{+}*Fcer1g*$^{-/-}$, and C57Bl/6 mouse—except for day 21 samples, when 4 strains were analyzed because of low survival in IIA,IIIB^{+}*Fcer1g*$^{-/-}$ animals. Data in which significant difference ($p < 0.001$) was shown by ANOVA were subjected to Tukey-Kramer for comparison between two mouse strains at 5% significance. In intravital microscopy, Mann-Whitney U test was used for analysis, and statistical significance was accepted at $p < 0.05$.

SUPPLEMENTAL DATA

Two figures and three movies are available at http://www.immunity.com/cgi/content/full/28/6/833/DC1/.

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